R76-582

Reprinted from
SYMPOSIUM ON ENZYMATIC HYDROLYSIS OF CELLULOSE
Aulanko, Finland 12-14 March 1975

PILOT PLANT INVESTIGATIONS OF TRICHODERMA VIRIDE CELLULASE PRODUCTION

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SUMMARY

The submerged fermentation of *Trichoderma viride* for production of cellulase has been successfully carried out on a pilot scale. The problems of foam formation have been alleviated through the use of both mechanical and chemical foam control. A reproducible fermentation profile has been established by standardization of inoculum size and age. The initial lag time in the batch fermentation has been reduced to 1 to 2 hours following inoculation. The rather complex behavior of the fermentation is explained as the result of the complex media and condition of the inoculum.

Results indicate low oxygen transfer requirements, even at the maximum rates of cell respiration. Substantial enzyme production has been observed in as little as 24 hours, indicating that maximum cellulase yields may require a fermentation of only 3 to 4 days.

INTRODUCTION

The production of consistently high quality cell-free cellulase enzyme on a large scale is a major objective of the Natick pilot program for enzymatic hydrolysis of waste cellulose. Laboratory production of enzyme broths *via* submerged fermentation of *Trichoderma viride* has been successfully studied by several investigators (1, 2, 3). Transferral of this laboratory technology to pilot scale operation is the first step towards a commercial process.

The goal of a pilot program is to develop successful processing

technology and to gather sufficient engineering data for a total process evaluation. For enzyme production it is first necessary to establish a reproducible fermentation profile or kinetic behaviour under a given set of operating parameters. Only after this is accomplished is it possible to evaluate the effect of individual parameters and to proceed towards more optimum conditions, or to investigate more sophisticated processing strategies.

Economics of a cellulose to glucose process have been proposed and questioned (4, 5, 6). There is serious concern over the cost of pretreatment of the substrate and some question regarding the economic production and recovery or reuse of the enzyme. This paper only begins to address one of these questions - the large scale production of enzyme.

EQUIPMENT AND METHODS

In order to obtain the data necessary for a good process evaluation a small pilot or pre-pilot facility has been assembled. For the production of enzyme a two-vessel system is used. A 30 liter fermentor (15 or 20 liter working volume) is employed as a "seed" or inoculum vessel for the larger 400 liter (300 liter working volume) production fermentor. Both vessels are equipped with Waldhof type draught tubes and pumping impellers.

The instrumentation for monitoring and controlling the vessels includes temperature, pressure, weight, pH, dissolved oxygen, power, agitation rate, sparge, and inlet and exit gas analysis for oxygen and carbon dioxide. Foam sensing and control by either automatic addition of surfactants or mechanical foam breakers is also a capability of both vessels.

Because of the problems of measuring growth on an insoluble substrate, three indirect methods are employed as indicators of growth or viable cell mass. The first is direct oxygen utilization which is calculated using inlet and exit gas analysis under controlled

sparge conditions. The second is a dynamic oxygen uptake parameter (K) calculated under conditions of zero sparge. This technique measures the rate of decline of dissolved oxygen between the 50 and 20 percent saturation levels. Dissolved oxygen is initially raised to 65 percent saturation before the sparge is turned off and the agitation rate is set at 250 rpm. The time for decline in dissolved oxygen between the 65 and 50 percent levels is sufficient to eliminate transients caused by bubble entrainment. Because of the standard agitation conditions, surface aeration is fairly constant for each experiment. The rate of decline of dissolved oxygen is invariably linear between the 50 and 20 percent levels, and the slope ($K = \Delta DO / \Delta TIME$) is used as an indicator of respiration and viable cell mass. This method is used only as a relative indicator for it does not take into account any probe factor. The third technique is the calculation of carbon dioxide production, again using inlet and exit gas analysis under controlled sparge conditions.

Enzyme production is measured in filter paper units with the analysis decribed in reference (7).

FERMENTATION PROCEDURES

Culture and media

Trichoderma viride was chosen as the best organism on the basis of the extensive work of Mandels *et al.* (1, 8). A mutated strain, QM-9414, has been selected because of its increased enzyme productivity over the parent or wild strain QM-6A.

The media used contained, in grams/liter, KH_2PO_4 2.0, $(NH_4)_2SO_4$ 1.4, $CaCl_2$ 0.3, urea 0.3, and $MgSO_4 \cdot 7H_2O$ 0.3 with 1% cellulose (SW40), and 0.075% proteose peptone. In addition, 65 ppm of polypropylene glycol (M.W. 2050) was added as an antifoam. Tap water was used, eliminating the need for trace metal addition. The media and vessel were sterilized at 121°C for 60 minutes.

The inoculum for the 30 liter vessel was prepared in the laboratory using the mineral salts medium (1) with 0.5% cellulose (SW40), 0.05% proteose peptone and 0.2% Tween 80 (Atlas Chemical Industries, polyoxyethylene sorbitan mono-oleate). This medium was inoculated with a spore suspension and grown at 30°C on a reciprocating shaker in Fernbach flasks.

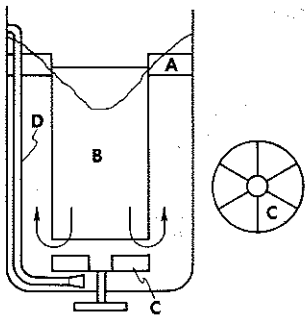
Foam control

The production of foams in fermentation media is a common (9) and sometimes serious problem. It can limit very seirously the working volume of a vessel. The escape of foam has the additional consequence of wetting exhaust air filters and lines with the subsequent threat of contamination. Denaturation of proteins or enzymes due to the stresses associated with bubble formation can be a serious problem when an enzyme is the desired product. There are other numerous disadvantages of uncontrolled foam formation which make it a primary consideration in the scale up of a fermentation (10).

Foaming in the complex media for the *T. viride* fermentation is due to several factors. The partially hydrolyzed proteins in the peptone are known for their foam enhancing properties. The entrapment of cellulose fibers within the foam yields a dry, stable froth, which is quite difficult to handle. Tween 80 has been used to "lighten" or to promote drainage of the foam on a laboratory scale (1). However, levels normally used are in 2000 ppm range. Sorbitan derivatives are not used at this high a level in commercial fermentations and generally the water soluble ones like Tween 80 are known to promote foam rather than to control it (10). For the large scale *T. viride* fermentation, Tween 80 was found to cause a major foaming problem even at mild agitation and aeration rates, and because of this it has been eliminated from the media.

A secondary foaming problem appears later in the fermentation with the appearance of high levels of soluble protein, of which a good portion is undoubtly the cellulase enzyme complex.

The Waldhof draught tube shown in Figure 1 has been effective in controlling foam in our large scale fermentation. In addition, polypropylene glycol in concentrations up to 65 ppm has been extremely effective as an antifoaming agent and has replaced Tween 80. It was selected, after trying many commercially available antifoams, for its long term effectiveness. A mechanical foam breaker located in the vessel head space is also used for back-up foam control.



A Foam deflector
B Draught tube
C Pumping impeller (bottom drive)
D Sparger

Fig. 1. Waldhof draught tube.

Inoculum

Though inoculum size does not seem to affect ultimate enzyme yield (1) it most certainly affects the rate of production. Initial runs were made using a 1% shake flask inoculum in the seed fermentor. As can be seen in Figure 2, it was soon evident that it would be impossible to obtain consistent results because of the varying lag time. This made it very difficult to predict optimum transfer time. A larger (10%) inoculum was tried with encouraging results. It is not uncommon to run industrial fermentations with inocula up to 20% in size (11, 12). This therefore is not a new innovation but simply an applied processing technique. As can be seen, the lag is reduced to around 1...2 hours and the peak culture respiration activity is higher and achieved in a period of hours instead of days. In addition the results have been surprisingly consistent. The culture activity profile can easily be reproduced and has been

the same (±2 h) for every run to date.

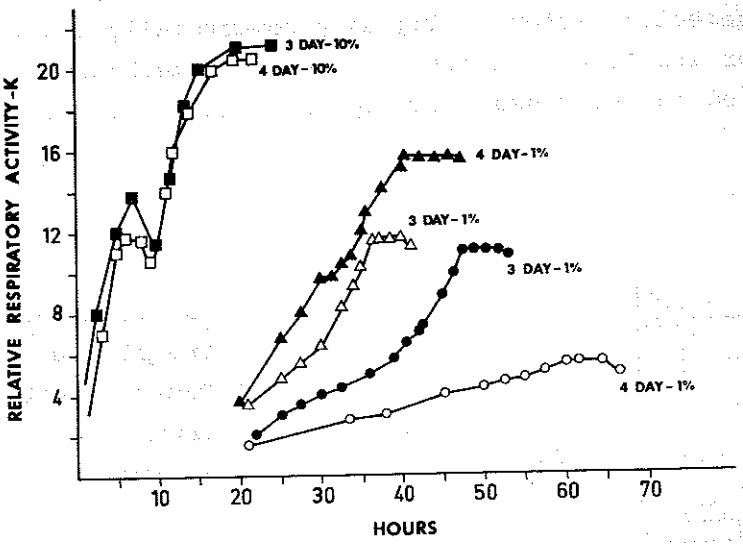


Fig. 2. Respiration activity profiles for *Trichoderma viride* fermentation. (Temperature 30°C, pressure 0.04 atm gage).

Run No.	Inoculum age	Inoculum size
3	3 days	1%
4	3 days	1%
5	4 days	1%
6	4 days	1%
8	4 days	10%
9	3 days	10%

The age of the inoculum was thought to have an effect on the lag and ultimate activity of the culture. Upon investigation it was found that 3 to 4 day old spore-inoculated shake flasks produce the best inocula (13). Both 3 and 4 day old flasks have been used in our pilot studies and little if any difference can be seen in their performance.

Fermentation profile

The conditions of the fermentation for production of *T. viride* and

T. viride cellulase have yet to be established at their optimum. At present, the temperature is controlled at 30°C ±0.1°C and vessel pressure at 0.04 atm gage.

In the 30 liter vessel an agitation rate of 360 rpm and sparge of 0.097 vvm give an average value for the volumetric adsorption coefficient ($k_L a$) of 50 millimoles O_2/l·h·atm and is sufficient to maintain dissolved oxygen levels in excess of 15% of saturation at the highest respiration rates. The pH is uncontrolled in the 30 liter vessel.

In the production vessel, an agitation rate of 200 rpm and a sparge of 0.05 VVM is sufficient to match the 30 liter oxygen transfer performance. The pH is controlled once it falls to 4.1 and is maintained between 4.1 and 4.15 by addition of 4N NaOH.

As previously mentioned, the profile is most definitely a function of inoculum size and age and with variations caused by these factors eliminated, the profile has a rather interesting story to tell.

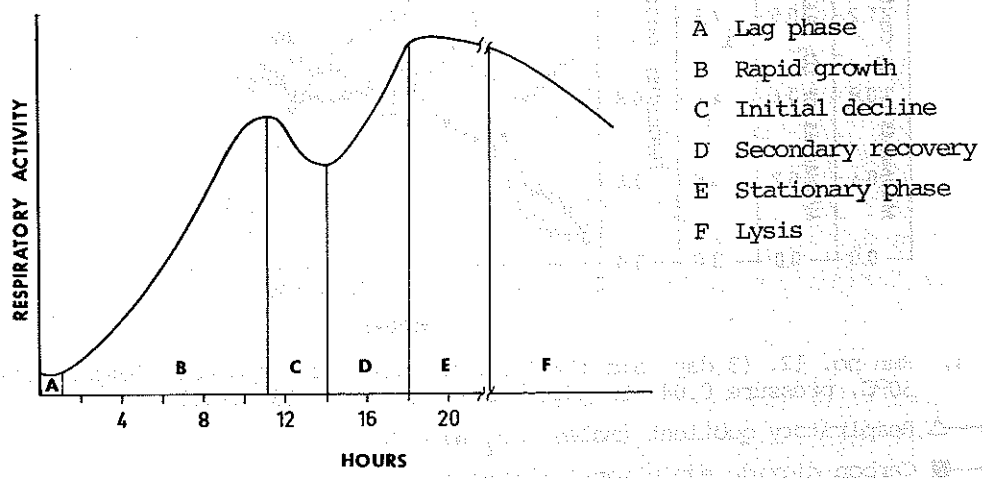


Fig. 3. General fermentation profile for 10% inoculum.

Using a 10% inoculum, the fermentation can be divided into several phases as depicted in Figure 3. There is a very small lag experienced during the first one or two hours followed by a very rapid growth phase. This is followed by a rather abrupt peak and decline to a lower level of activity. The fermentation recovers from this decline, rising to a new peak level of activity which is maintained for several hours. The final decline is characteristic of an aging fermentation and there is no recovery. Though this behaviour is somewhat complex it is consistent from run to run. This behavior is reflected in the four selected parameters shown in Figure 4, for an inoculum being prepared in the "seed" vessel. The carbon dioxide production and oxygen consumption follow the general profile shown in Figure 3, while the pH and respiratory quotient indicate shifts in metabolism at various stages in the fermentation. It is presently thought that transfer of the culture should take place between the 16th and 24th hours, just prior to or during the time of peak cellular activity.

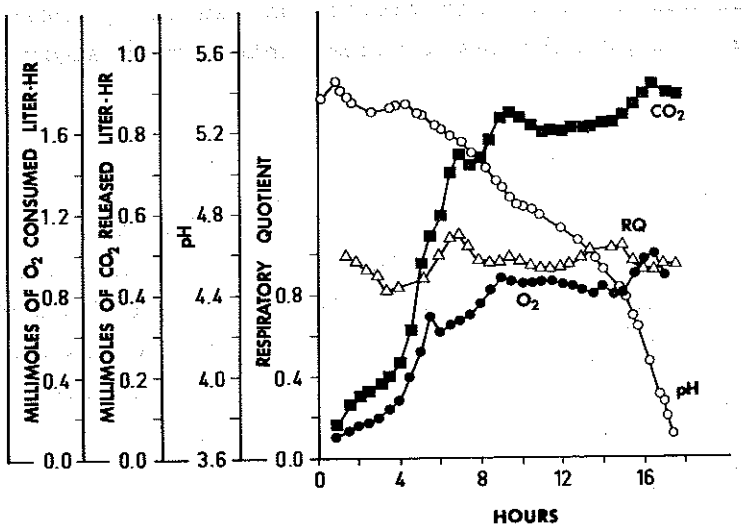


Fig. 4. Run no. 12. (3 day old 10% inoculum grown from spores. Temperature 30°C, pressure 0.04 atm gage, aeration rate 0.097 vvm.)

△—△ Respiratory quotient (moles CO_2/mole O_2)

■—■ Carbon dioxide evolution (millimoles O_2/l·h)

○—○ pH

●—● Oxygen uptake (millimoles/l·h)

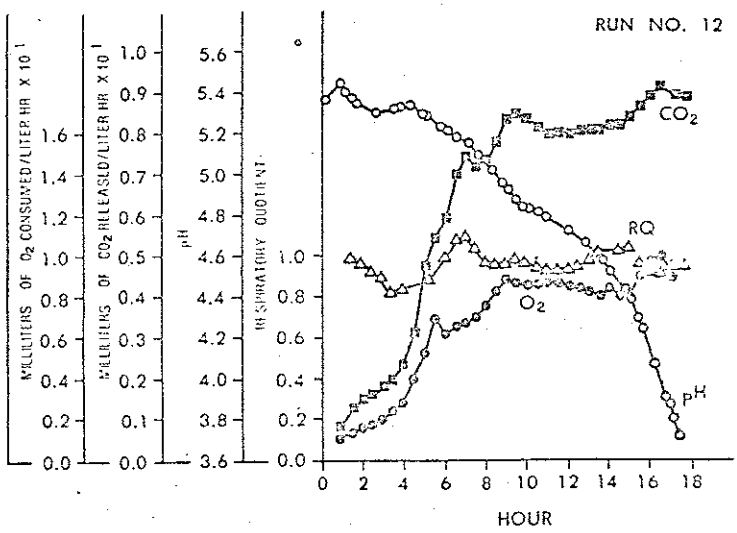


FIGURE 4

Run No. 12
10% Inoculum
Inoculum grown from spores (Age - 3 days)
Temperature = 30.0°C Pressure = 0.04 atm gage
Aeration Rate = 0.097 VVM

Respiratory Quotient moles CO_2/mole O_2

Carbon Dioxide Evolution milliliters O_2/1hr x 10^{-1}

pH

Oxygen Uptake - milliliters CO_2/1hr x 10^{-1}

DISCUSSION

The complex fermentation profile is a result of the complex media and the condition of the inoculum. The initial lag is common to all fermentations and as mentioned before can be controlled by the inoculum size. The initial rapid growth rate is not characteristic of growth on cellulose. In this case it is probably due to two factors. First, the proteins in the peptone may be the most readily available substrate. Their consumption is noted by a rise in pH due to deamination. Secondly, the inocula are characteristically high in cellulase activity and it is not long before there is a substantial level of cellulose hydrolysis and production of reducing sugars. Growth becomes very rapid and a shift in respiratory quotient is noted as metabolism shifts from the proteins to the carbohydrates.

As previously mentioned, the growth rate is abnormally high and it soon approaches the rate of sugar production. The level of cellular activity peaks and then declines due to the suddenly limited sugar substrate. The pH which was falling very rapidly during the high prowth period levels off as deamination of peptone proteins or perhaps cell lysis begins. This is reflected again in a shift in respiratory quotient.

The culture recovers from this shock and maintains itself at a somewhat lower level of activity. It is postulated that enzyme production is stimulated at this point and correspondingly hydrolysis rates increase. Culture activity increases, and there is again a shift in respiratory quotient and an acceleration in pH decline. Eventually a steady state is approached where hydrolysis and sugar utilization rates are matched.

Further increase in cellular activity becomes cellulose substrate limited. The more amorphous fraction has been depleted and the rate limiting step becomes the action of cellulase on the more recalcitrant cellulose fractions.

Little is assumed about cellulase production. It has been previously postulated that it appears in high levels only after the rapid growth period but preliminary results of this pilot study show heavy production during the first 16 to 24 hours. Rapid growth does not seem to inhibit its production. Table 1 shows results from four separate runs with high and low levels of cellulase activity determined in the inoculum. However, overall performance of all the cultures seems to be comparable. There is room for wide speculation but no conclusions will be drawn on this scant amount of data. There is, however, a strong suggestion that cultures with cellulase activities as low as 1.4 filter paper units may make excellent inocula and that this level of enzyme activity may be achieved in as little as 19 to 20 hours.

Table 1.

Run no.	FPA of inoculum	Transfer time hours	FPA of transfer
7	3.72	24	1.95
9	1.44	28	1.96
10	2.20	16	1.06
12	3.18	17	1.06

FPA - Filter paper activity

An additional point should be made at this time. Very few successful production runs (400 liter scale) have been made to date. There have been serious problems with contamination which have only recently been resolved. However, the most recent results indicate the same fermentation profile as experienced in the "seed" vessel.

CONCLUSIONS

1. The control of foam formation during the fermentation can be accomplished by both mechanical means and antifoam agents. A Waldhof draught tube when used with polypropylene glycol has given excellent foam control on the 400 liter scale.

2. A $k_L a$ of only 50 millimoles $O_2/l \cdot h \cdot atm$ is necessary to maintain a sufficient dissolved oxygen level even during peak cellular activity.

3. Inoculum size greatly affects the rate of enzyme production. A 10% inoculum can reduce the previous 1...2 day lag time to 1...2 h.

4. The fermentation profile is complex due to the complex media but has to date been totally reproducible.

5. Rapid growth does not seem to inhibit enzyme production. Substantial filter paper assays can be obtained in less than 24 hours. Ultimate production of the enzyme may require only 3 to 4 days for maximum cellulase yields.

6. Initial indications are that an optimum transfer time may be between 16 and 24 hours.

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